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TCRβ Transmembrane Tyrosine Tyrosines Are Required for Pre-TCR Function

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The pre-TCR promotes thymocyte development in the αβ lineage. Productive rearrangement of the TCRβ locus triggers the assembly of the pre-TCR, which includes the pTα chain and CD3 εγδζ subunits. This complex receptor signals the up-regulation of CD4 and CD8 expression, thymocyte proliferation/survival, and the cessation of TCRβ rearrangements (allelic exclusion). In this study, we investigate the function of two conserved tyrosine residues located in the TCRβ chain transmembrane region of the pre-TCR. We show that replacement of both tyrosines with alanine and expression of the mutant receptor in RAG-1<sup>-/-</sup> thymocytes prevents surface expression and abolishes pre-TCR function relative to wild-type receptor. Replacement of both tyrosines with phenylalanines (YF double mutant) generates a complex phenotype in which thymocyte survival and proliferation are severely disrupted, differentiation is moderately disrupted, and allelic exclusion is unaffected. We further show that the YF double mutant receptor is expressed on the cell surface and associates with pTα and CD3ε at the same level as does wild-type TCRβ, while association of the YF double mutant with CD3ζ is slightly reduced relative to wild type. These data demonstrate that pre-TCR signaling pathways leading to proliferation and survival, differentiation, and allelic exclusion are differentially sensitive to subtle mutation-induced alterations in pre-TCR structure.

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in T hybridomas and mature primary spleen T cells (22, 23). Mutation of either transmembrane Y or both to leucine or alanine disrupts receptor assembly, greatly reducing coexpression and signaling (22). Others have shown that mutation of the C-terminal conserved transmembrane tyrosine to leucine disrupts human TCR assembly with CD3ζ, and when Jurkat T lymphoma cells are selected that express high levels of the TCR lacking CD3ζ, they have reduced apoptotic responses to Ag (24–27).

Although tyrosines can be sites of phosphorylation, the residues under examination in this study are embedded in a lipid bilayer and are unlikely kinase substrates. They are more likely to be sites of protein-protein interactions. Consistent with this possibility, mutation of the relatively polar tyrosine side chains to nonpolar residues, alanine, leucine, or phenylalanine, was disruptive; if no protein contacts were made such mutations would be expected to be more energetically favorable within the bilayer than the native tyrosine residues. In addition, it has been recently shown that polar asparagine residues within an engineered hydrophobic transmembrane domain promote protein homodimerization within the bilayer (28–30).

Receptor complex assembly, followed by signal transduction involving protein tyrosine kinase and other pathways, are critical features of pre-TCR function (1). There are both similarities and differences between pre-TCR and mature TCR assembly and signaling. If TCRβ transmembrane tyrosine mutations affect signaling mechanisms in common between mature and pre-TCR receptors, we would expect they might affect thymocyte development. This prediction is confirmed herein by our findings that TCRβ mutations cause defects in pre-TCR function. Mutation of one or both transmembrane tyrosines to leucine or alanine prevents pre-TCR surface expression on Scid.adh cell lines and abrogates pre-TCR function and T cell development. Replacement of both tyrosines with phenylalanines allows surface expression but expresses a complex developmental phenotype in which allelic exclusion is unaffected, CD4−8+ differentiation is reduced, and thymocyte expansion is severely impaired.

Materials and Methods

Mice

RAG-1null C57BL/6 inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred for timed pregnancies in our facility in sterile housing. Recipient thymic lobes were obtained from C57BL/6 or B10.BR mice obtained from The Jackson Laboratory and bred in our facility. Animal use protocols are reviewed annually by the Institutional Animal Care and Use Committee.

Retroviral constructs

TCRβ mutations were made using site-directed mutagenesis as described previously (22, 23). Mutants were shuttle to the MIGR-1 vector (gift of W. Pear, University of Pennsylvania, Philadelphia, PA) and confirmed by sequencing. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use. Retroviral supernatants were generated by transient transfection of Phoenix cells (31) and titered on 3T3 cells before use.

Cells, Abs, and flow cytometry

Immature thymoma cells derived from SCID mice, Scid.adh, were provided by D. Wiest (Fox Chase Cancer Center, Philadelphia, PA). mAbs were used as follows: biotinylated KJ25 specific for TCR Vβ3 (BD PharMingen, San Diego, CA), PE-conjugated anti-TCRβ H57–59 (Caltag Laboratories, Burlingame, CA), PE-conjugated anti-CD3e 2C11 (Caltag Laboratories), alphaprotocyanin-conjugated anti-CD8 (BD PharMingen), biotinylated anti-CD4 (Caltag Laboratories), streptavidin red 670 (BD Biosciences, Mountain View, CA), streptavidin PerCP (BD PharMingen), PE-conjugated anti-CD25 (BD PharMingen), PE-conjugated anti-CD5 (BD PharMingen), and PE-conjugated anti-Vβ8 (F23.1; BD PharMingen). Anti-CD3ε Y (mAb 7D6) was a gift of L. Samelson (National Institutes of Health, Bethesda, MD). CD3ζ-specific HMT 3–1, TCRα-specific H16–C10, and pTα-specific polyclonal Abs were gifts from D. Wiest. Surface staining was done in 96-well plates using standard procedures. For DNA analysis, cells were surface stained as required, and after final wash they were fixed for 30 min on ice in freshly made 0.5% paraformaldehyde in PBS. Cells were centrifuged and resuspended in 0.1% Triton X-100 in PBS for 3 min, washed once more in PBS, resuspended in 1 μM 4′,6-diamidino-2-phenylindole (DAPI) in PBS, incubated 1 h overnight in the dark, and analyzed. Analysis was performed on BD Biosciences instruments, including FACScan, FACSCalibur, and LSR, and analyzed using FlowJo software according to standard procedures.

Fetal liver infection and FTOC

Rag-1null mice were set up for breeding and checked for vaginal plugs daily for 4 days. Plugged females were sacrificed (day 4). Fetuses were harvested on days 14–16 and disrupted, and cells were frozen in FCS/10% DMSO. Cell suspensions were thawed, washed, and plated at 2 × 105 cells/ml with 10% X supplement (source of IL-3; cells were the gift of F. Melchers, Basel Institute for Immunology, Basel, Switzerland) and 1% stem cell factor (PeproTech, Rocky Hill, NJ) in RPMI 1640 complete (10% FCS, gentamicin, glutamine, 5 × 10−5 2-ME, 10 mM HEPES, pH 7.2). Recovered cells after 5 days of culture (24 h is generally equal to 2 days) were harvested, washed, and replated in 24-well plates at 105 cells/well, plus 0.5 μl retroviral supernatant, polybrene (6 μg/ml), and 20% X, 1% stem cell factor. Virus was pelleted onto cells by spinning plates at 700 rpm for 50 min at room temperature. After 24–36 h, cells were harvested, washed, and resuspended at 105–106 cells/ml for 30 to 40 μl. A total of 35 μl of cell suspension was placed into well–sized wells of a 96-well plate (Nunc, Rochester, NY). Recipient thymic lobes were irradiated at 2500 rad, and one lobe was added to each well. Plates were inverted and placed into a plastic box with wet paper towels pre-equilibrated in incubator. After 24 h, each lobe was transferred to standard fetal thymic organ culture (FTOC) conditions, fed weekly, and analyzed after 14–20 days.

Immunoprecipitation and recapture assay

Immunoprecipitation and Western blotting were conducted as described elsewhere (23). In summary, Scid.adh cells were isolated by Ficoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation followed by several washes in PBS. Cells (1 × 107) were lysed in lysis buffer containing digitonin as detergent and then precipitated using protein A-agarose beads prebound to the designated Ab. Protein separation and immunoblotting were conducted using standard techniques using appropriate Abs and visualized with HRP-bound protein A and chemiluminescence.

For recapture assays, Scid.adh cells were washed twice with HBSS and labeled with biotin for 30 min on ice, after which cell viability was consistently ≥97%. After labeling, the cells were lysed at the density of 5 × 107/ml for 20 min on ice in buffer containing 1% digitonin (high purity; Wako Pure Biochemicals, Osaka, Japan). The lysates were preclarified at 4°C for 1 h with protein A-Sepharose beads (Amersham Biosciences). The extracts were then immunoprecipitated for 2 h at 4°C with the anti-TCRβ (H57-597) mAb prebound to protein A-Sepharose. The resultant immune complexes were washed three times with 0.2% digitonin washing buffer and once with PBS. The beads were boiled for 5 min in 100 μl of 1% SDS and the SDS–eluted proteins were quenched with 900 μl of 1% Nonidet P-40 lysis buffer. The solution containing the solubilized proteins was re-immunoprecipitated as above with anti-pTα cytoplasmic tail Ab (gift of D. Wiest). The recaptured immune complexes were resolved on 12% SDS–PAGE, transferred to membrane, and visualized with HRP-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL).

Results

We tested the abilities of a collection of TCRβ transmembrane mutants to rescue the early steps of T cell development in RAG-1null thymocytes. We investigated the two conserved tyrosines embedded in the transmembrane domain of TCRß. Tyrosines have large, polar side chains, so to test these features we made replacements with alanine and leucine (small, nonpolar) and phenylalanine (large, nonpolar). RAG-1null fetal liver cells were infected by retroviral vectors encoding the 2B4 TCRß chain wild type (WT), or encoding TCRß with mutations of the conserved tyrosines: Y to A at both positions 265 and 275 (YA double mutant (YA DM)),
and Y to F at both positions (YF DM) (Fig. 1). We also tested Y to A, L, and F mutations at the C-terminal tyrosine (275). The vector used (MIGR1) contains the enhanced GFP marker expressed from an internal ribosome entry site. Infected fetal liver cells include T cell progenitors and were used to repopulate irradiated thymic lobes in FTOC. After 14–20 days, lobes were harvested and analyzed by flow cytometry.

RAG-1null fetal liver cells infected with the empty vector control generated thymocytes blocked at the CD4+8− (DN) stage, with very low thymus cellularity, similar to uninfected controls (Table I). The WT TCRβ-containing retrovirus increased thymocyte numbers by >10-fold compared with vector-only control (Table I). There was an increase in the percentage of GFP+ cells in the WT transductants relative to vector control, indicating a positive selection for GFP- and TCRβ-expressing thymocytes during thymus repopulation. GFP+ thymocytes expressing the WT receptor were found to express both CD4 and CD8 consistent with pre-TCR function and further development (Fig. 2 and Table I). CD25 is expressed at high levels in immature thymocytes from RAG-1null mice and also in thymocytes infected by the vector-only control (Table I and Fig. 2). WT TCRβ expression on RAG-1null thymocytes induced differentiation as shown by a decrease in the percentage of thymocytes expressing CD25 and a decrease in the density of CD25 expression on both DN and DP thymocytes (Table I and Fig. 2).

In contrast, substitution of both tyrosines with alanines (YA DM) failed to rescue thymocyte development (Table I and Fig. 2). The small gains in thymic cellularity observed for YA DM were not statistically significant, and the percentage of GFP+ thymocytes was similar to vector control, indicating that there was no expansion of YA mutant TCRβ-expressing progenitors (Table I). CD4 and CD8 expression was not induced (Fig. 2), and CD25 expression remained high (Fig. 2). Similarly, substitution of a single Y at 265 and 275 (YF DM) caused a distinct phenotype, as described below.

The YF DM TCRβ mutants were less able to restore thymocyte expansion relative to WT TCRβ. The average number of thymocytes recovered from YF DM samples was only 32% of WT controls (Table I). The percentage of DP thymocytes in YF DM samples was also reduced, although less severely, compared with WT (66% of control; Fig. 2 and Table I). YF DM thymocytes also showed higher levels of CD25 expression on DN and DP thymocytes, relative to WT (Fig. 3 and Table I). In addition, CD5 was expressed at lower levels on YF DM thymocytes compared with WT (Fig. 3). Taken together, these changes in expression indicate that there is a defect of maturation in the cells expressing the mutant TCRβ. Some of the differences observed between mutant and WT TCRβ could be related to the reduction in proliferation. It has been suggested that proliferation is required for the down-modulation of surface molecules such as CD25 (5).

To further explore the underlying basis of reduced thymocyte expansion, we examined proliferation directly in subpopulations of thymocytes using DAPI staining for DNA content. In RAG-1null thymocytes expressing WT receptor, the most actively cycling subpopulation was CD4+CD25+GFP+, in which ~30% of thymocytes were in the S/G2 phases of the cell cycle. In contrast, only ~20% of CD4+CD25+GFP+ thymocytes from YF DM-TCRβ or vector control infected thymuses were in S/G2 (Fig. 4A). All other populations cycled at the rate of 10% or less, and the percentage of cycling thymocytes did not differ between WT and vector control (data not shown).

We also determined the fraction of dead cells in reconstituted thymic organ cultures. There was a statistically significant increase in 7-amino actinomycin D (7-AAD)-positive cells in the YF DM samples compared with WT (Fig. 4B). Taken together, these experiments provide evidence for a reduction in both proliferation...
and survival contributing to the reduction in cellularity of YF DM-expressing thymuses.

There was no correlation between the percentage of DP and the number of thymocytes recovered for either WT or mutant samples (Fig. 5). Therefore, up-regulation of CD4 and CD8 expression during thymocyte development is not necessarily linked to thymocyte survival/proliferation. The development of DP thymocytes without expansion has been observed previously, in situations where both distal (p53 deficiency (5), Ikaros deficiency (32)), and proximal (Gads deficiency (33)) signaling mediators are disrupted. Therefore, TCR signals controlling DP differentiation vs proliferation/survival are partially distinct and likely bifurcate early in the signaling pathway.

The pre-TCR induces allelic exclusion at the TCR/locus, preventing the expression of dual TCR specificities. To test whether TCRβ mutations could affect this function, we infected normal fetal liver (RAG-1/H11001) and reconstituted irradiated FTOCs. To determine whether the YF DM receptor was capable of allelic exclusion, we tested for the expression of endogenous TCR/ receptors (the frequently occurring Vβ8 family) on the surface of GFP+ thymocytes. As expected, we found that Vβ8 was expressed on vector control infected thymocytes (Fig. 6). Vβ8 receptor expression was suppressed on GFP+ cells of both WT and YF DM infected thymocytes, indicating that the YF DM pre-TCR is effective at mediating allelic exclusion (Fig. 6).

We next used the GFP marker to determine whether pre-TCR function could be correlated with TCR/ expression level. Low levels of the TCR/ expression on DP thymocytes, together with relatively high background staining of the anti-Vβ3 reagent on thymocytes from organ culture, precluded the possibility of making direct measurements of TCRβ expression. However, the MIGR1 vector expresses both the TCR/ and the GFP marker protein from a single IRES-containing message driven by the murine stem cell virus retroviral promoter/enhancer. These vectors are susceptible to regulation by the chromosomal site of integration (position effects), leading to interlobe variations in GFP.
expression levels. We took advantage of these variations to determine whether there was a correlation between high levels of GFP expression—and therefore TCRβ—and other developmental parameters.

There was no correlation between GFP means and the number of thymocytes recovered from YF DM infected thymic lobes (Fig. 7A). These experiments suggest that quantitative differences in pre-TCR expression, over the range we observed, are unable to overcome the inability of YF DMs to support thymocyte expansion. In contrast, there was a correlation between GFP expression and the formation of DP thymocytes. We divided the WT and YF DM infected populations into three groups based on GFP fluorescence intensity, low (<200), intermediate (200–1000), and high (>1000). We determined the GFP mean fluorescence intensity (MFI) and the percentage of DP thymocytes in each subdivided population, and we computed a correlation coefficient for the data (Fig. 7B). We found a high degree of correlation ($r = 0.56$, $p < 0.001$ confidence level) between GFP MFI and the percentage of DP thymocytes recovered for both the WT (data not shown) and YF DM (Fig. 7B) infected populations.

We also examined this question conversely. If the failure of YF DMs to make DP thymocytes was in part due to mutation-induced reductions in TCRβ expression or a quantitative reduction in the signal induced by mutated receptors, we might expect there to be a selection for higher levels of GFP intensity among YF DM DPs compared with WT DPs. This prediction is confirmed when the data are first gated on the DP subpopulation and the GFP means are calculated. YF DM-bearing DP cells have a MFI of GFP expression significantly higher than that of WT DP cells (GFP MFI 674 for YF DM and 482 for WT; significantly different by t test, $p < 0.001$).

These data indicate that, with respect to DP development, the YF DM phenotype results from a quantitative reduction in pre-TCR signaling, due to either a reduction in surface expression of TCRβ or a reduction in signaling strength. To distinguish between these possibilities, we tested whether the mutants could be expressed on the surface of Scid.adh tumor cells, a cell line derived...
from SCID mice, which has been previously shown to express pT\(\alpha\) and other CD3 components required for pre-TCR assembly, but shown to lack all endogenous rearranged TCR chains (TCR\(\beta\) and TCR\(\alpha\)) (34). We found that the YF DM TCR\(\beta\) was indeed expressed on the cell surface of Scid.adh cells (Fig. 8A). Surface expression of YF DM TCR\(\beta\) was comparable to WT when detected with the pan-\(\beta\) monoclonal H57 (Fig. 8A). In addition, surface expression of CD3\(\varepsilon\) (2C11) and CD3\(\gamma\varepsilon\) (7D6) was also induced by YF DM as well as or better than WT receptors (Fig. 8A). These results indicate that the YF DM TCR\(\beta\) is expressed on the surface, likely in a complex with CD3\(\varepsilon\) and \(\gamma\). We determined the subunit composition of the pre-TCR complex by coimmunoprecipitation (Fig. 8B). We found that the CD3\(\varepsilon\) and \(\chi\) chains were coimmunoprecipitable with TCR\(\beta\) from both WT and YF DM, but not from uninfected Scid.adh lines. We noticed a slight reduction (up to 2-fold, relative to WT) in the ability of YF DM TCR\(\beta\) to coimmunoprecipitate with CD3\(\varepsilon\) in Scid.adh cells (Fig. 8B).

To determine whether the YF double mutations affected the association of TCR\(\beta\) with pT\(\alpha\), we performed recapture assays. Scid.adh cells from uninfected, WT, and YF DM clones were surface-biotinylated and immunoprecipitated using anti-TCR\(\beta\) Ab, and the complexes were resolubilized and precipitated with antibodies against pT\(\alpha\). Single bands of the appropriate size for pT\(\alpha\)/TCR\(\beta\) dimers were visualized by streptavidin-HRP staining of nondenaturing gel blots. We found that there was no reduction in the amount of pT\(\alpha\) associated with YF DM TCR\(\beta\) compared with WT (Fig. 8B).

**Discussion**

We have shown that YF DM mutations clearly affect the ability of the pre-TCR to mediate thymocyte developmental progression, proliferation, and survival. Although the pre-TCR is expressed at low levels on the cell surface normally, this low surface expression appears to be a requirement for function. We find no defect in surface expression of the YF DM pre-TCR in Scid.adh cells, suggesting that failure of surface expression alone cannot account for the YF DM defect. Therefore, although we have not yet directly shown any signaling defects in the YF DMs, we think it is likely that the YF DM mutations alter some aspect of pre-TCR signaling function.

CD3 subunits are clearly involved in signaling by the pre-TCR complex through conserved ITAM motifs. In the mature TCR, evidence supports a quantitative role for ITAM function. For example, it was shown in TCR transgenics that TCR specificity (and, presumably, affinity) could affect the ability of CD3\(\varepsilon\) transgenes which lack one or more ITAM to rescue CD3\(\varepsilon\) deficiency (35). Furthermore, the propensity for a given TCR to be positively or negatively selected on a given background correlated with the number of ITAMs present, where fewer CD3\(\varepsilon\) ITAMs appeared able to convert a negatively selecting signal to a positively selecting one. These previous experiments provide a quantitative link among TCR affinity, signaling strength, thymocyte selection, and the number of ITAMs, in the context of the mature TCR. Because the YF DM mutations affect the ability of CD3\(\varepsilon\) to associate with TCR\(\beta\), the phenotype we observe could be due to a reduction in signaling strength due to a reduction in the number of engaged ITAMs. Although coimmunoprecipitation experiments show only a very minor deficiency in CD3\(\varepsilon\) association, it is possible that functional coupling of the receptor to CD3\(\varepsilon\) is more highly affected. With respect to DP formation, overexpression of the mutant was able to alleviate the defect in function which supports a quantitative model of signaling. Our observations are the first to support the quantitative signaling model with respect to pre-TCR function.

In contrast, the survival and proliferation of thymocytes was not observed to be correlated with pre-TCR expression levels in YF DMs. This suggests that the YF DM pre-TCR may induce signals that differ from WT qualitatively as well as quantitatively. Recent data concerning mature TCR signaling are also inconsistent with a purely quantitative model of signaling (reviewed in Ref. 36). Specifically, it has been shown that mutations in the TCR\(\alpha\) connecting peptide motif prevent the association of CD3\(\delta\) in the mature TCR complex (37). The CD3\(\delta\)-less receptor of connecting peptide motif mutants (or CD3\(\delta\) knockout mice (38)) is expressed on the cell surface and is competent to signal for negative selection but does not mediate positive selection. Additional experiments showed that the requirement for CD3\(\delta\) in positive selection was independent of its ITAM sequence and correlated with the ability to activate extracellular signal-regulated kinase (37, 38). Future experiments will be aimed at discovering whether the YF DM TCR\(\beta\)-containing pre-TCR may also induce qualitatively different signals, which could explain its particular inability to support thymocyte proliferation and survival.

The pre-TCR functions by activation of multiple signaling pathways. For example, activated lek (39, 40) and protein kinase C...
(PKC) (41) were shown to be equivalent to pre-TCR in rescuing thymocyte development, while activated Ras and Raf restored proliferation, differentiation, and survival, but failed to induce allelic exclusion (42). Further studies showed that differentiation, proliferation, and survival mediated by activated lck required Rho activity, while lck-induced allelic exclusion was Rho independent (43, 44). Defects in thymocyte survival and proliferation by Vav activity, while proliferation and differentiation require PKC, Ras, Rho, and Rac pathways. In the case of the YF DM TCRγ, which is more defective for proliferation and survival than for differentiation or allelic exclusion, we speculate that engagement of any or all of the Ras, Rac, and Rho pathways may be reduced, while other PKC-dependent pathways are normally activated. Signaling strength could determine which pathways are activated if the intrinsic thresholds for activation of key components differ. In addition, there could be qualitative differences in YF DM signaling compared with WT, due to subtle structural alterations. Our experiments so far are consistent with both possibilities, but continued evaluation of the structure and function of YF mutants in thymocytes are likely to provide insights into the mechanism of pre-TCR signaling.

Acknowledgments

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